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Sequence-specific positioning of nucleosomes over the steroid-inducible MMTV promoter

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We have investigated chromatin organization over the MMTV LTR, a promoter regulated by steroid hormones. The studies were performed on cell lines containing BPV-based episomal constructs. Nucleosome positioning was determined by localization of sites sensitive to the enzyme micrococcal nuclease, or to the chemical MPE-Fe(II). Experiments with both reagents indicate that nucleosomes are specifically positioned in MMTV LTR chromatin. In the absence of hormone a regular cutting pattern is obtained, with cleavage sites at +136, −60, −250, −444, −651, −826 and −1019 relative to the Cap site. In the presence of hormone the cutting pattern is unchanged, except for a region between −60 and −250 that becomes hypersensitive to MPE-Fe(II). This region contains the DNA sequences to which steroid receptor complexes bind during transcriptional activation. Our results indicate that this region is associated in chromatin from uninduced cells with a macromolecular complex (probably a nucleosome core), and this complex is displaced (or modified) upon binding of activated receptor.

Key words: phasing /MMTV/steroid receptor/chromatin structure

Introduction

Packaging of the large quantity of DNA in eukaryotic cell nuclei is achieved by its association which histones into nucleosomes, and subsequent organization of the chromatin fiber into higher order structures (for reviews see Eissenberg *et al.*, 1985; Pederson *et al.*, 1986). The observation that nucleosomes can be positioned over specific DNA sequences, or phased (reviewed in Zachau and Igo-Kemenes, 1981; Eissenberg *et al.*, 1985), introduces the question of a potential regulatory role for such structures. Significant alterations in chromatin structure frequently accompany gene activation, and such changes (usually detected as DNase I hypersensitive regions, or HSRs) often occur in regions of the DNA that contain regulatory sequences (Eissenberg *et al.*, 1985). Specific positioning of sequences in polynucleosomal arrays could theoretically modulate the interaction of transacting regulatory proteins with their binding sites.

Evidence for nucleosome phasing *in vivo* was obtained initially from experiments with micrococcal nuclease, which preferentially attacks internucleosomal (linker) DNA. This approach is seriously compromised (McGhee *et al.*, 1983) by the observation that the enzyme does not cleave deproteinized DNA randomly (Dingwall *et al.*, 1981; Horz *et al.*, 1981) More recently, preferential cleavage of the linker DNA has been achieved using a chemical reagent, methydiumpropyl-EDTA-Fe(II) [MPE-Fe(II); Cartwright *et al.*, 1983], whose cleavage specificity for naked DNA is essentially random. Compelling evidence has also been advanced that nucleosomes can associate with specific DNA sequences *in vitro* (Simpson and Stafford, 1983; Ramsey *et al.*, 1984; Cereghini and Yaniv, 1984).

Here we report studies on chromatin organization of the hormonally regulated promoter from mouse mammary tumor virus (MMTV). The MMTV long terminal repeat (LTR) contains a regulatory element (HRE) that confers responsiveness to steroid hormones (Huang *et al.*, 1981; Lee *et al.*, 1981; Chandler *et al.*, 1983; Ponta *et al.*, 1985; Cato *et al.*, 1986). Sequences responsible for hormone induction correlate well with sites of preferential binding by purified glucocorticoid receptors (Scheidereit *et al.*, 1983; Payvar *et al.*, 1983), indicating that receptor binding is the critical event in promoter activation. Activation of the promoter is accompanied by the acquisition of a DNase I HSR over the receptor binding sites (Zaret and Yamamoto, 1984; Hager *et al.*, 1984), indicating a localized change in cleavage structure attendant to hormone induction.

To facilitate characterization of nucleoprotein structure at the MMTV LTR, we utilized cell lines containing amplified extrachromosomal chimeras. We previously demonstrated that functional MMTV promoters can be mobilized on bovine papilloma virus (BPV) based episomes (Ostrowski *et al.*, 1983). These chimeras replicate stably in mouse cells at a high copy number (50−300 copies/cell), and support glucocorticoid regulated transcription (Ostrowski *et al.*, 1983).

We have determined cleavage positions of MPE-Fe(II) and micrococcal nuclease in MMTV chromatin, both from uninduced and hormone-treated cells. Cutting sites were localized by the indirect end-labelling method (Nedospasov and Georgiev, 1980; Wu, 1980). Experiments with both reagents indicate that nucleosomes are specifically positioned on MMTV LTR sequences. The sites to which purified glucocorticoid receptor binds are found to be protected from nuclease and chemical cleavage in the absence of hormone. Upon hormone treatment, a modification of the chromatin structure in this region leads to the generation of a hormone-dependent hypersensitive site. The position of this HSR correlates well with the inferred position of one nucleosome in the LTR phased array.

Results

Episomal amplification of the regulated promoter

To facilitate detailed studies of the nucleoprotein organization of the MMTV promoter and associated regulatory sequences, we developed cell lines containing the MMTV LTR mobilized on bovine papilloma virus (BPV)-based episomes. Maps for constructions used in the studies described here are shown in Figure 1. Properties of cell lines containing episomes pm18 and pm19 have been described previously (Ostrowski *et al.*, 1983). Episome pm23 was obtained by inserting the SV40 small-t antigen intron and early region polyadenylation signal downstream of the v-ras^H gene in pm19 (see Materials and methods). Cell lines

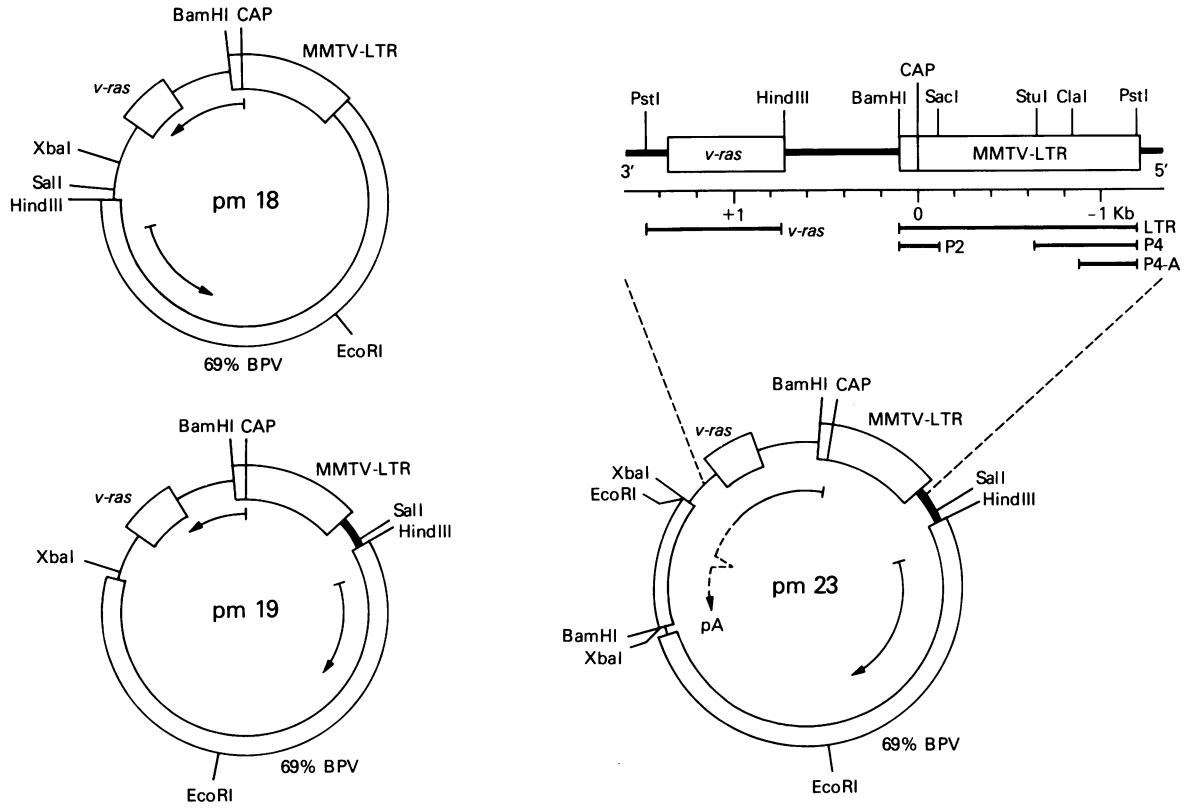

Fig. 1. Maps of BPV-MMTV episomal chimeras. Maps describe the structure of the episomal chimeras replicating in NIH3T3 and C127 cells. Episomes pm18 and pm19 have been described previously (Ostrowski *et al.*, 1983). Episome pm23 contains the SV40 small-t splice site and early region polyadenylation site, inserted at the *Xba*I site downstream of the v-ras^H coding sequence. Details of the MMTV LTR-v-ras^H cassette, common to each of the three episomes, are presented in the upper right portion of the figure. Probes used to analyze chromatin digestion patterns are described below in detail. The LTR probe extends from *Pst*I (−1179) to *Bam*HI (+105), P4 probe from *Pst*I (−1179) to *Stu*I (−636), P4-A probe from *Pst*I (−1179) to *Cla*I (−860), P2 probe from *Sst*I (−104) to *Bam*HI (+105) and v-ras^H probe from *Hin*dIII (+750) to *Pst*I (+1490). Coordinates are measured from the MMTV LTR Cap site (Donehower *et al.*, 1981). Arrows inside the construct maps indicate the direction of transcription from the MMTV and BPV promoters.

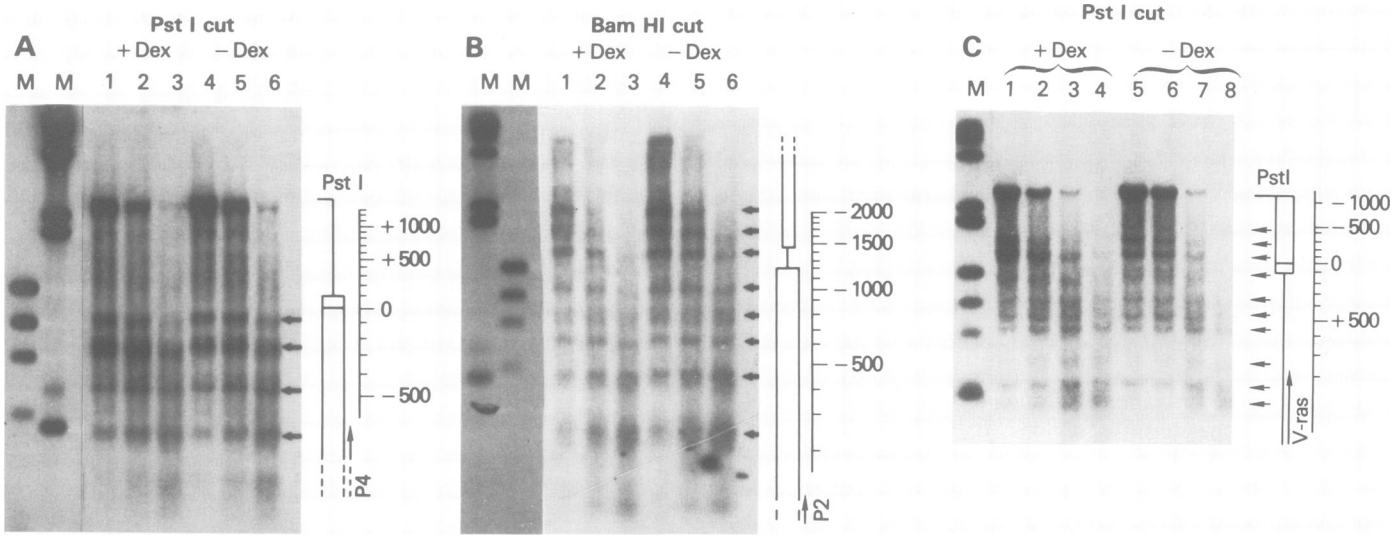

Fig. 2. Micrococcal nuclease chromatin digestion patterns. **Panel A.** *Pst*I-restricted DNA hybridized with P4 probe. Nuclei from 1361.5 cells (pm23), treated or untreated with dexamethasone 0.1 μM for 4 h, were digested for 5 min at 25°C with micrococcal nuclease. Micrococcal nuclease concentrations were 30 units/ml **(lanes 1** and **4)**, 60 units/ml **(lanes 2** and **5)**, or 120 units/ml **(lanes 3** and **6)**. The purified DNAs were digested to completion with the indicated restriction enzymes, 20 μg DNA was electrophoresed on 1.5% agarose gels, and the DNAs transferred to nitrocellulose membranes. Marker lanes (M) contained a mixture of λ DNA restricted with *Hin*dIII and φX174 DNA restricted with *Hae*III. A map of sequences resolved in each panel is presented at the right of the autoradiograms. Distances are measured from the MMTV LTR Cap site. **Panel B.** *Bam*HI-restricted DNA hybridized with P2 probe. DNAs and micrococcal nuclease conditions were the same as in panel A. **Panel C.** *Pst*I-restricted DNA hybridized with v-ras^H probe. DNAs were the same as panel A. Micrococcal nuclease concentrations were 30 units/ml **(lanes 1** and **5)**, 60 units/ml **(lanes 2** and **6)**, 120 units/ml **(lanes 3** and **7)**, or 225 units/ml **(lanes 4** and **8).**

904.13 and 935.1-11 are transformants of C127 cells; each cell contains ~200 copies of episome pm19 and pm18, respectively. Cell line 1361.5 is a transformant of NIH3T3 cells, and contains 200 copies/cell of episome pm23. In each of these cells, the LTR-v-ras^H fusions replicate uniquely as non-rearranged episomes, and transcription of v-ras^H sequences is highly responsive to glucocorticoid induction (see Ostrowski *et al.*, 1983). The structure of the LTR-v-ras^H cassette common to each of the episomes is detailed in Figure 1.

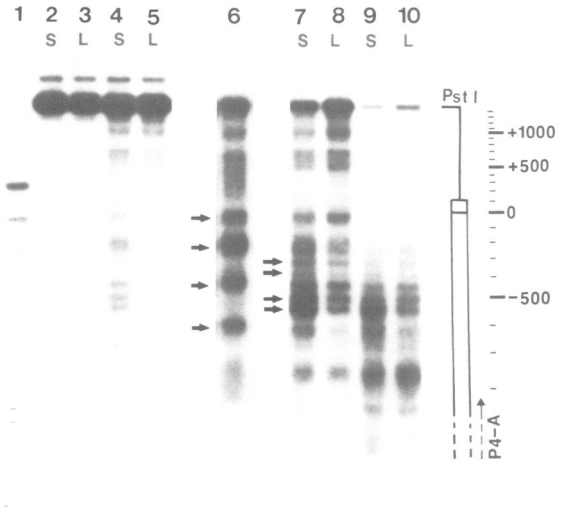

Fig. 3. Micrococcal nuclease digestion of deproteinized DNA. Plasmid pM19 DNA, either supercoiled (S) or linear (L) was digested with micrococcal nuclease as described in Materials and methods. Digested DNAs were purified, restricted to completion with *Pst*I and electrophoresed (100 ng/lane) on 2% agarose gels; the DNA was transferred to nitrocellulose membranes and the filters hybridized with P4-A probe; **lanes 2** and **3**, no micrococcal nuclease; **lanes 4** and **5**, 0.075 units; **lanes 7** and **8**, 0.15 units/ml; **lanes 9** and **10**, 0.75 units/ml. **Lane 6** contained 5 μg DNA from nuclei of cell line 1361.5 (pm23) digested with 120 units/ml of micrococcal nuclease. **lane 1** contained marker ϕX174 DNA restricted with *Hae*III.

Micrococcal nuclease analysis of episomal chromatin

Episomal DNA sequences were first characterized for cleavage periodicity with micrococcal nuclease. Initial experiments (data not shown) indicated that the minichromosomes were organized in nucleosomal arrays. Potential sequence specific, or 'phased', localization of nucleosomes was investigated by positioning the micrococcal-sensitive, linker sequences with the indirect end-labelling technique (Nedospasov and Georgiev, 1980; Wu, 1980). Minichromosome-containing nuclei were subjected to varying extents of micrococcal nuclease digestion. After deproteinization and restriction endonuclease digestion, DNA fragments were analyzed with directional probes (see Figure 1). Figure 2 presents an analysis of the MMTV LTR region in pm23 episomes from 1361.5 cells. In panel A, the DNA was digested with *Pst*I before electrophoresis and the blot hybridized with P4 probe. The intense band at the top of the autoradiogram is the parental *Pst*I fragment, uncut by the micrococcal nuclease, that includes the complete LTR v-ras^H cassette (Figure 1). A series of LTR-specific fragments generated by micrococcal nuclease cleavage correspond to cleavage sites at positions −65, −250, −450 and −640 relative to the MMTV Cap site. In panel B, the samples were digested to completion with *Bam*HI and hybridized with P2 probe, extending the analysis into the upstream LTR and adjacent BPV regions; cleavage positions in the LTR were detected at −255, −465, −660, −820, −1050, −1400, −1700 and −2100. Positions were also measured from the *Pst*I site in the v-ras^H gene (panel C). These data are included in the summary of preferential cleavage sites presented in Figure 8.

The digestion of MMTV LTR chromatin by micrococcal nuclease clearly results in a nonrandom cleavage pattern, with a

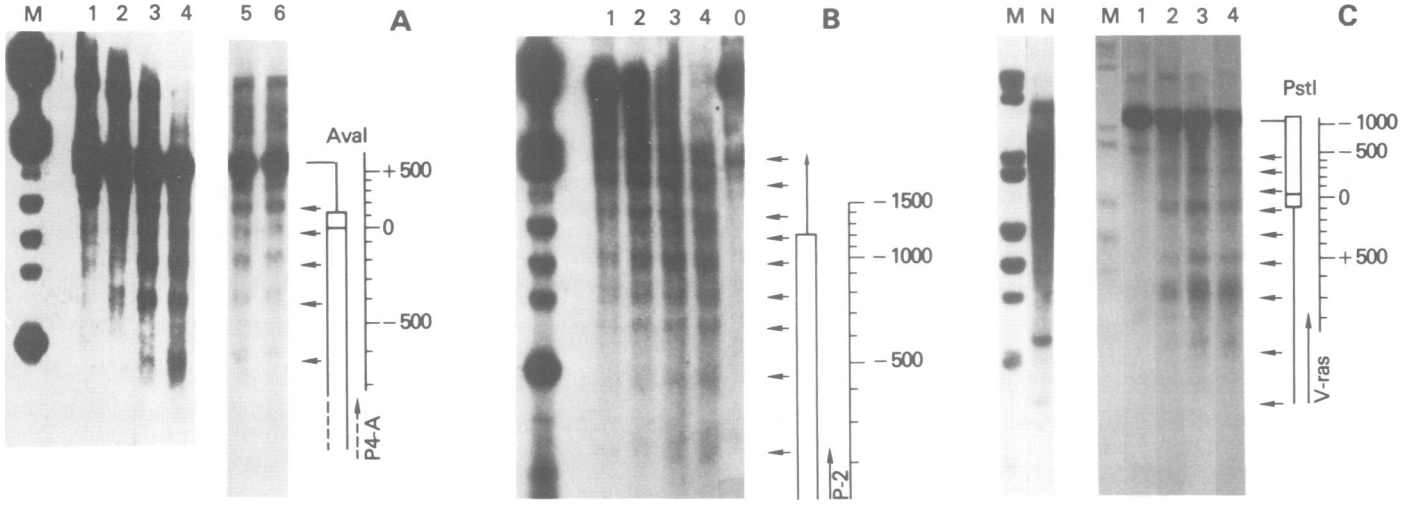

Fig. 4. MPE-Fe(II) chromatin cleavage patterns. MPE-Fe(II) cleavage sites were analyzed in chromatin from the 1361.5 cell line (pm 23). Nuclei were diluted to an OD_{260} of 15 and treated with MPE-Fe(II) as described in Materials and methods. The cleavage reactions were performed in the presence of 2 mM H_2O_2. After MPE-Fe(II) cleavage DNAs were purified from the nuclei and digested with the appropriate restriction enzyme prior to indirect end-labelling analysis (20 μg DNA/lane, 2% gels). Indirect end-labelling analysis was performed with the restriction enzyme and probe combinations indicated. Marker lanes (M) contained a mixture of λ DNA cut with *Hin*dIII and ϕX174 DNA cut with *Hae*III. A map of sequences resolved in each panel is presented at the right of the autoradiograms. Distances are measured from the MMTV LTR Cap site. **Panel A.** *Ava*I-restricted DNA hybridized with P4-A probe. Cleavage times at 25°C were: 10 μM MPE-Fe(II); (**lane 1**, 0 min; **lane 2**, 5 min; **lane 3**, 20 min; **lane 4**, 30 min) 25 μM MPE-Fe(II): (**lane 5**, 40 min; **lane 6**, 60 min). **Panel B.** *Bam*HI-restricted DNA hybridized with P2 probe. Nuclei were treated with 10 μM MPE-Fe(II); H_2O_2 was not present during the cleavage reactions for this panel. Cleavage times at 25°C were: **lane 0**, 0 min. **lane 1**, 15 min. **lane 2**, 30 min. **lane 3**, 45 min. **lane 4**, 60 min. **Panel C.** *Pst*I-restricted DNA hybridized with v-ras^H probe. Nuclei were treated with 25 μM MPE-Fe(II). Cleavage times at 25°C were: **lane 1**, 0 min. **lane 2**, 20 min; **lane 3**, 40 min. **lane 4**, 60 min. The bands visible in lane N are endogenous ras sequences, visible because of the longer exposure. The lane designated N contains DNA from 1361.5 cells deproteinized prior to treatment with MPE-Fe(II), as described in Materials and methods.

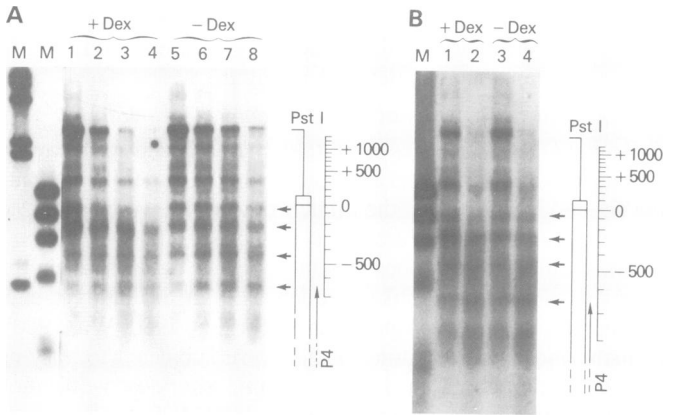

Fig. 5. Vector and cell-line independence of micrococcal nuclease cleavage pattern. **Panel A.** Cell line 903.14 (pm18). Nuclei were isolated from cells either treated or untreated with micrococcal nuclease for 5 min at 25°C. After digestion with *Pst*I, 20 μg DNA was electrophoresed on 2% agarose gels, transferred to nitrocellulose membranes and the filters hybridized with P4 probe. Micrococcal nuclease concentrations were: **lanes 1** and **5**, 30 units/ml; **lanes 2** and **6**, 60 units/ml; **lanes 3** and **7**, 120 units/mol; **lanes 4** and **8**, 225 units/ml. Marker lanes (M) contained *Hin*dIII-cut λ and *Hae*III-cut φX174 DNAs. **Panel B.** Cell line 935.1-11 (pm19). Ten μg *Pst*I-digested DNA was applied to each lane. Micrococcal nuclease concentrations were: **lanes 1** and **3**, 75 units/ml; **lanes 2** and **4**, 225 units/ml.

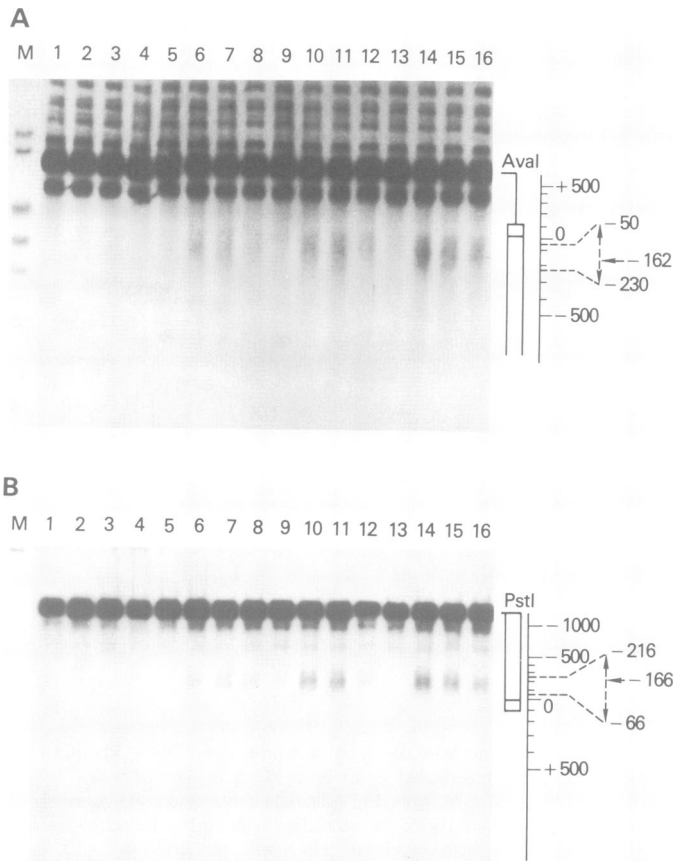

Fig. 6. Hormone-dependent DNase I-hypersensitivity of MMTV LTR. **Panel A.** *Ava*I-restricted DNA hybridized with P4-A probe. Cell line 904.13 was treated for 1 h with different concentrations of dexamethasone: **lanes 1, 5, 9** and **13**, no hormone; **lanes 2, 6, 10** and **14**, 100 nM; **lanes 3, 7, 11** and **15**, 20 nM; **lanes 4, 8, 12** and **16**, 7 nM. Nuclei were isolated in buffer containing the same concentrations of hormone, diluted to an OD_{260} of 20, and treated for 10 min at 37°C with DNase I: **lanes 1–4**, no enzyme; **lanes 5–8**, 3 units/ml; **lanes 9–12**, 4 units/ml; **lanes 13–16**, 6 units/ml. After purification, 20 μg DNA was digested to completion with the indicated enzyme, and electrophoresed on 1.5% agarose gels. The DNA was transferred to a Nytran membrane, and the membrane hybridized to P4-A probe. Marker lanes (M) contained a mixture of λ DNA cut with *Hin*dIII and φX174 DNA cut with *Hae*III. A map of sequences resolved in each experiment is presented at the right of each panel, and the boundaries of the DNase I hypersensitive region are indicated. Distances are measured from the MMTV LTR Cap site. **Panel B.** *Pst*I-restricted DNA hybridized with v-ras^H probe. DNA was limit digested with *Pst*I and hybridized to v-ras^H probe.

spacing varying between 180 and 200 bp. This suggests the presence of specifically localized, or phased, nucleosomes in MMTV LTR chromatin. Since micrococcal nuclease can exhibit nonrandom digestion patterns with deproteinized DNA (Dingwall *et al.*, 1981; Horz and Altenburger, 1981), the enzyme's cleavage specificity for the MMTV LTR primary DNA sequence was examined. The results (Figure 3) indicate that micrococcal nuclease exhibits a strong primary sequence specificity for the MMTV LTR. The cleavage pattern with DNA is irregular, however, lacking the strict periodicity found for sequences organized in chromatin. In particular, the most sensitive sites in naked DNA, located at positions −520 and −560, are completely suppressed in chromatin (compare lane 6 with lanes 7−10, Figure 3). Naked DNA cleavages at positions −320 and −390 are also suppressed in chromatin. We conclude that preferential cleavage sites in chromatin represent a subset of sites with intrinsic increased sensitivity to the enzyme. This pattern is imposed on DNA by some feature of chromatin, most likely the presence of phased nucleosomes.

MPE-Fe(II) cleavage of chromatin

The non-random cleavage pattern of micrococcal nuclease for MMTV LTR naked DNA led us to analyze nucleosome positioning with another reagent. Treatment of nuclei with low amounts of methidiumpropyl-EDTA-FE(II) results in specific chemical cleavage of linker DNA (Cartwright *et al.*, 1983). Figure 4 presents an analysis of MMTV LTR chromatin, from cells untreated with the hormone, when probed with MPE-Fe(II). Indirect end-labelling analysis of the MPE-Fe(II) cleavage product was performed with the following enzyme cleavage and hybridization probe combinations: panel A, *Ava*I and P4-A; panel B, *Bam*HI and P2; panel C, *Pst*I and v-ras^H.

Two major findings are apparent. First, for each of the combinations, a periodic series of preferential cleavages by MPE-Fe(II) is observed (indicated by arrows at the side of each panel in Figure 4, and summarized in Figure 8). The repeat distance in this series varies between 180 and 200 bp. Secondly, over the complete LTR, these cleavage positions correlate well with the sites of preferential micrococcal digestion reported above. Furthermore, the presence of a cleavage site at position −60 indicates that the cut observed in this region with micrococcal nuclease is not due to preferential cutting of naked MMTV LTR DNA by the enzyme at this position.

Vector independence of chromatin cleavage sites

Unique positioning of nucleosomes in MMTV LTR chromatin could theoretically result from boundary conditions created by the vector sequences immediately adjacent to the LTR, rather than specific association with LTR DNA. To address this question, we examined nucleosome phasing on the LTR with the vector in reverse orientation. In this configuration, a potential boundary contributed by the vector DNA would be at a consider-

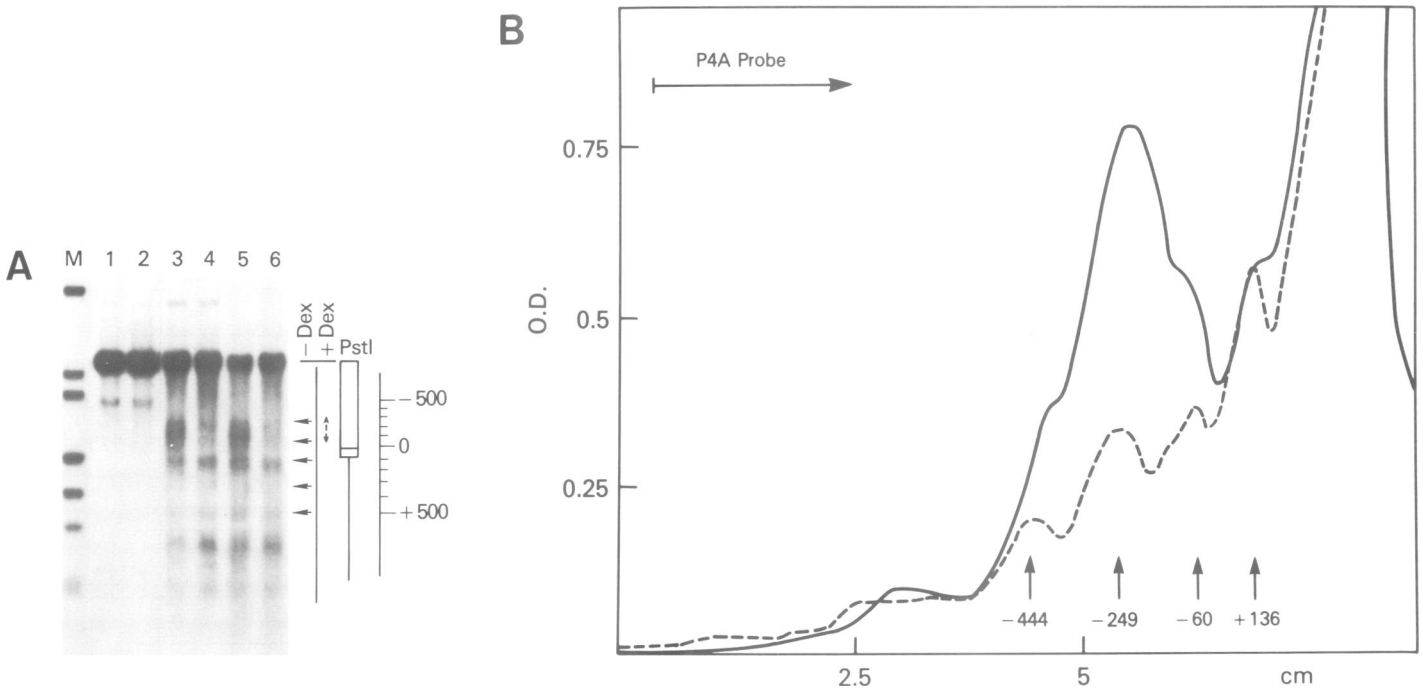

Fig. 7. Hormone-dependent MPE-Fe(II) cleavage. Cell line 1361.5 (pm23) was treated **(panel A, lanes 1, 3, 5)** or untreated **(lanes 2, 4, 6)** with dexamethasone (0.1 μM) for 1 h. Nuclei were isolated in buffer containing the same concentrations of hormone, diluted to an OD_{260} of 15, and treated at 25°C with MPE-Fe(II) at a final concentration of 25 μM, in the presence of H_2O_2 (see Materials and methods). MPE-Fe(II) treatment was carried out for 0 min **(lanes 1** and **2)**, 30 min **(lanes 3** and **4)** or 60 min **(lanes 5** and **6)**. DNAs were purified, digested to completion with *AvaI* or *PstI*, electrophoresed on a 2% agarose gel, transferred to a Nytran membrane, and the membranes hybridized respectively with the indicated probe. **Panel A.** Autoradiogram of the blot hybridized with v-ras^H probe. The marker lane (M) contained a mixture of λ DNA restricted with *Hin*dIII and φX174 DNA digested with *Hae*III. Sequences resolved in the gel are diagrammed at the right. **Panel B.** Densitometric scan of an autoradiogram after hybridization with P4-A probe. Conditions were identical to lane 3 (panel A, + DEX) (—), or lane 4 (panel A, − Dex) (----). The numbers indicate the cutting site positions. Distances are measured from the MMTV LTR Cap site.

able distance from the LTR, and also out of phase relative to the original construction. The results are shown in Figure 5. In panel A, micrococcal nuclease-sensitive sites are shown for episomal LTR chromatin in cell line 904.13 (episome pm18, C127 parental cell), using the P4 probe and *PstI* digestion. In panel B, the same analysis is shown for cell line 935.1-11 (episome pm19, C127 parental cell). LTR orientation in 935.1-11 is the same as 1361.5, but the episome is replicating in a different cell; 904.13 contains the opposite LTR orientation, also in C127 cells. It is readily apparent from these data that internucleosomal cleavage sites are independent of LTR vector orientation and cell line. These sites have now been examined in several cell lines with varying episomal constructions; we never observed a significant deviation from the pattern presented here. We conclude that adjacent vector sequences do not contribute significantly to the positioning of nucleosomes, and that LTR phasing is an intrinsic property of the DNA sequence.

Hormone dependence of MPE-Fe(II) and DNase I hypersensitivity

Transcriptional activation at the MMTV promoter has been shown to coincide with the acquisition of a DNase I-hypersensitive site (Zaret and Yamamoto, 1984; Hager *et al.*, 1984). This site coincides with sequences to which the glucocorticoid receptor binds *in vitro*. We have confirmed the presence of this hormone dependent DNase I-hypersensitive site in episomal chromatin. Figure 6 documents the hypersensitivity dose-response by indirect end-labelling analysis with cell line 904.13 (pm18). Whether probed from the left end of the LTR (panel A), or from the direction of the *ras* gene (panel B), a broad region of hypersensitivity (150−180 bp) is observed in LTR chromatin. This region is centred 165 bp upstream of the Cap site, and includes both receptor binding sites observed *in vitro*.

We also examined hormone-dependent changes in sensitivity to MPE-Fe(II) attack. Figure 7 shows an analysis of MPE-Fe(II) cleavage in pm23 chromatin (cell line 1361.5) after transcription induction with hormone. Superimposed on the periodic cleavage pattern observed in the absence of hormone (Figure 7, panel A, lanes 4 and 6; see also Figure 4), a region of MPE-Fe(II) hypersensitivity develops in the region between the first (−60) and second (−250) LTR cleavage sites (panel A, lanes 3 and 5, and panel B). Thus, hormone stimulation of the episomal promoter results in a chromatin modification such that sequences between −60 and −250 become sensitive both to DNase I and MPE-Fe(II).

Discussion

Nucleosome phasing on the MMTV LTR

The *in situ* MPE and micrococcal nuclease digestion studies reported here demonstrate that nucleosomes are specifically positioned over the MMTV promoter and associated regulatory regions. Comparison of digestion patterns of deproteinized DNA and chromatin indicates nucleosome phasing from position +136 to the 5′ end of the LTR (nucleosomes A to F in Figure 8). Micrococcal data for the 3′ end of the LTR is inconclusive, since cutting patterns of deproteinized and chromatin DNA are very similar. These uncertainties were resolved through the use of MPE-Fe(II), a chemical reagent that also preferentially cuts the linker region in the chromatin, but does not display a cutting specificity for DNA primary sequence. MPE-Fe(II) cleavage produced essentially the same cutting patterns as micrococcal nuclease (Figure 8).

No major changes in the micrococcal cleavage pattern are associated with hormone stimulation, indicating that transcrip-

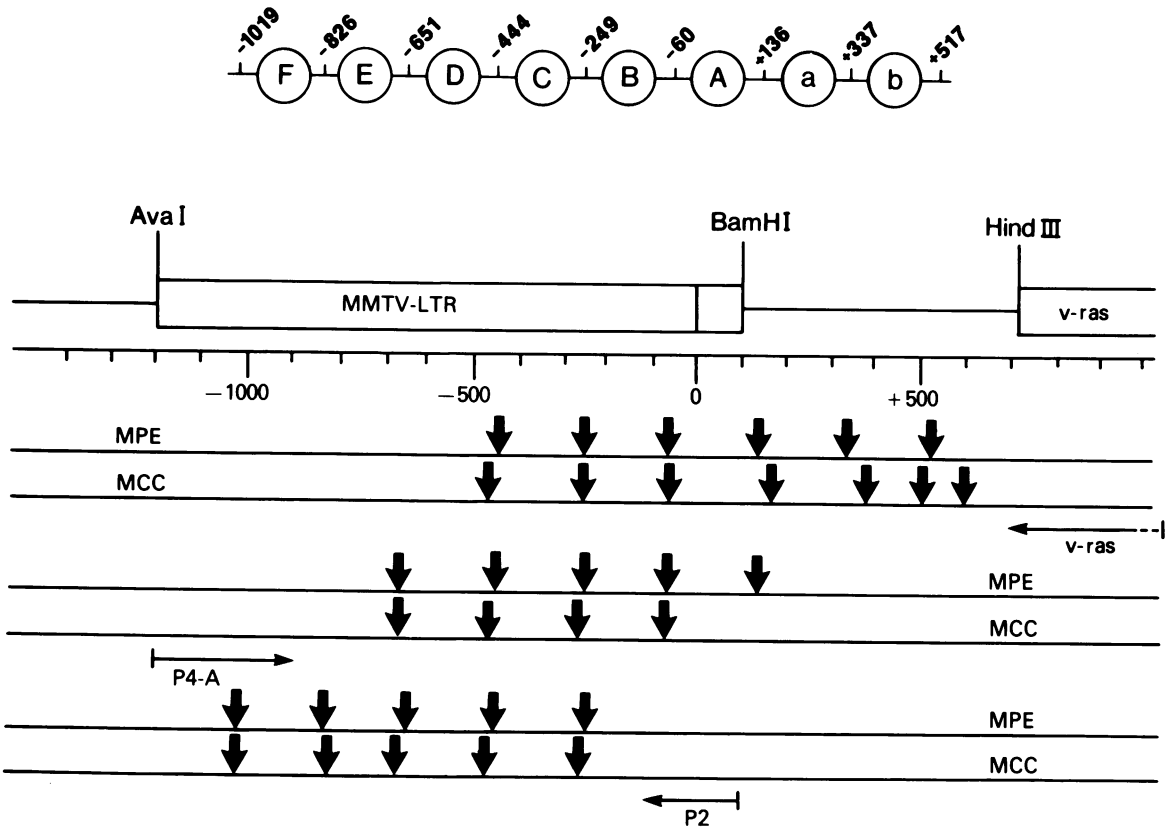

Fig. 8. Summary of micrococcal nuclease and MPE-Fe(II) cleavage data. Preferential cleavage sites in the MMTV LTR and v-ras^H chromatin are summarized for each of the probes. Arrows represent the mean center position of the cutting sites obtained from several digestion or cleavage experiments. For micrococcal nuclease digestion, data were averaged from six experiments with the v-ras^H probe, seven experiments with the P4-A probe, and two experiments with the P2 probe. For MPE-Fe(II) cleavage, the positions were determined from three experiments in each case. The proposed nucleosome positioning is presented at the top of the figure, and the midpoint of the internucleosomal linker region, obtained from the chemical cleavage data, is indicated.

tional activation is not accompanied by a complete rearrangement of LTR chromatin structure. However, a specific region of the LTR (−60 to −250) becomes hypersensitive both to MPE-Fe(II) and DNase I. This area of hypersensitivity, which includes both glucocorticoid receptor binding sites, corresponds precisely to sequences putatively associated with nucleosome B. The other micrococcal and MPE-Fe(II) cutting sites are unaffected by hormone stimulation, suggesting that the major chromatin structural alteration is localized to the receptor binding domain. Although the whole LTR is more susceptible to micrococcal nuclease attack in hormone activated chromatin (lanes 2 and 6 in Figures 2A and 3B), the HSR is not readily detected by this enzyme. This is not unexpected, given the resistance to micrococcal digestion of deproteinized LTR DNA in the −100 to −200 region (Figure 3).

We conclude that nucleosomes are associated with specific sequences in the MMTV LTR. Since the hormone-dependent hypersensitive site we detected in minichromosomes is identical in location and kinetics of appearance to the site described for the LTR integrated in cellular DNA, we infer that features of chromatin organization visualized with the BPV-LTR chimeras apply to the proviral state as well. These findings raise several points of interest.

Nature of the hypersensitive site

Our results show that in chromatin from uninduced cells, an area of MMTV LTR DNA is resistant to attack by DNase I, the chemical reagent MPE-Fe(II), as well as restriction endonucleases (Cordingley and Hager, unpublished observations). This region becomes selectively accessible to each of these reagents upon activation of the hormone−receptor complex. The dose-response of the hypersensitive site closely parallels occupancy of the glucocorticoid receptor by hormone ligand, arguing that site formation is a direct result of receptor binding.

The most straightforward model for formation of the hypersensitive site is simply displacement of nucleosome B from the phased array as a result of hormone-receptor complex binding. In developmentally regulated systems, it has been argued that nucleosome-free regions are maintained by the presence of high affinity DNA-binding proteins that prevent nucleosomes from associating (Emerson and Felsenfeld, 1984; Brown, 1984). In these systems, one can postulate that stage-specific transcription factors could bind to the DNA immediately after replication and prevent nucleosome deposition. In the MMTV system, since DNA replication is not required for hormone activation, the nucleosome would have to be actually displaced by hormone-receptor complex. In this regard, we note the experiments of Stein *et al.* (1985). These investigators suggest that core octamer can transiently associate with other nucleosomes, indicating that nucleosome dissociation could occur with only a small expenditure of energy, allowing the rapid reconstitution of the nucleosome.

It is possible that the nucleosome undergoes a structural modification, rather than actual displacement. *In vitro* reconstitution experiments have demonstrated that transcription factor TFIIIA can be associated with H1 depleted nucleosomes to form ternary complexes (Rhodes, 1985). Under such conditions, TFIIIA appears to displace nucleosomal DNA over a short region, although the ramifications of this event for *in vivo* hypersensitivity are not

described. The steroid hormone receptor complex could induce a similar structural alteration, loosening of the DNA-nucleosome interaction such that nucleosomes associated DNA became more accessible (see Prior *et al.*, 1983 for one possible model).

An alternative model would invoke protection of DNA in the hypersensitive region by nonhistone DNA-binding proteins, rather than a nucleosome core. With this mechanism, the correlation in position and size of the protected region with putative nucleosome B would be fortuitous. Activated hormone-receptor complex would compete for the region with factor(s) that protect the region from nucleosome and chemical attack.

We have recently shown (Cordingley *et al.*, 1987) that a high-affinity transcription initiation complex (composed of NF-1 and at least one other factor, F-i) is assembled at the MMTV promoter in response to hormone activation. In these experiments, the Exonuclease III footprinting technique was utilized to detect proteins bound to LTR chromatin. It was found that factors present in the nucleoplasm with high DNA binding affinity were nonetheless excluded from LTR chromatin in non-induced cells. No evidence was obtained for tight-binding proteins in the HSR region in instimulated chromatin. These experiments are inconsistent with the model that nonhistone high-affinity DNA binding protein(s) are present over the HSR (HRE) region in instimulated cells. If such proteins exist, they must be labile to displacement by Exonuclease III.

Mechanism of hormone action

Although the LTR acquires a specific and reproducible nucleoprotein structure *in vivo*, there is no direct evidence that chromatin structure is critical to the mechanism of hormone action. It has been argued (Ptashne, 1986) that the activity of *cis*-acting enhancers, even when placed at a considerable distance from the target promoter, can be adequately explained purely on the basis of protein−protein interactions. Under this view, interactions between receptor and other transcription factors would critically determine the sequence of events upon hormone induction. The concomitant nucleosome core displacement (or modification), could in fact be the basis of the observed hypersensitivity, but would occur as a result of the receptor−DNA interaction, rather than playing a direct role in promoter activation.

Several consequences of specific nucleosome positioning should be noted, however. In the absence of topoisomerase activity, nucleosome removal would be accompanied by a local change in superhelical density. Such an alteration in DNA structure could affect binding of other components of the transcription initiation complex. Binding sites for transcription factors could also be directly masked by positioning of the octamer core. We note in this regard the apparent exclusion of NF-1 (a high affinity DNA binding protein) from its MMTV promoter site in uninduced chromatin (Cordingley *et al.*, 1987). Whatever the mechanism responsible for development of the HSR in the MMTV regulatory sequences, it is unlikely that the receptor−template interaction can be completely modelled with pure receptor protein and DNA. We believe the ultimate resolution of these issues awaits reconstruction of the hormone response in cell-free systems, either with the purification of chromatin templates assembled *in vivo*, or with appropriate nucleoprotein substrates assembled *in vitro*. The isolation and characterization of the MMTV-BPV episomal chimeras will be of considerable utility in this effort.

Materials and methods

Plasmids, episomes and cell lines

Episomes pm18 and pm19, and cell line 935.1-11 (containing pm19) have been described previously (Ostrowski *et al.*, 1983). Cell line 904.13, obtained by transformation of C127 cells, contains 200 copies of pm18. Plasmid pM23 was constructed by inserting an *Eco*RI fragment containing the modified SV40 small-t intron and polyadenylation site from pSV2-CAT at the *Xba*I site downstream from the v-ras^H coding sequence in pM19. Deletion of pBR322 sequences between the two *Sal*I sites, followed by transfection of NIH3T3 cells, gave rise to episome pm23, and cell line 1361.5. Acquired BPV-MMTV-ras sequences replicate uniquely as non-rearranged episomes in each of the cell lines, and initiation rates at the episomal MMTV promoters, determined by run-on transcription assays, were induced ~25-fold by glucocorticoids.

Nuclei preparation

Cells were grown to confluence in roller bottles (2×10^8 cells/bottle), treated for 1 h with or without 0.1 μM dexamethasone, scraped and collected by centrifugation. All subsequent operations were performed at 0°C and all buffers used for treated cells contained 0.1 μM dexamethasone. Cell pellets were resuspended (10 ml/roller bottle) in chilled homogenization buffer (10 mM Tris−HCl, pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2% Nonident P-40, 5% sucrose) and lysed in a Dounce homogenizer (10 strokes — pestle A). The homogenate was centrifuged for 20 min at 1600 × *g* through a 10% sucrose in homogenization buffer cushion. The pellets were resuspended in wash buffer (10 mM Tris−HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine), pelleted again and resuspended with 2 ml/roller bottle of wash buffer. The amount of DNA in the nuclear suspensions was estimated by measuring the absorbance at 260 nm of a fraction of the nuclear suspension lysed in 1% SDS.

Micrococcoal nuclease digestion

The cold nuclear suspension was distributed in 1 ml fractions, adjusted to 1 mM $CaCl_2$ and held on ice. The reaction was initiated by the addition of various amounts of the enzyme (PL Biochemicals, 15 KU/ml) and the tubes incubated at 25°C. After 5 min, the reaction was stopped by addition of 100 μl of 100 mM EDTA, 10 mM EGTA, pH 7.5 mixture. The samples were adjusted to final concentrations of 1% SDS, 12.5 mM EDTA, 100 μg/ml proteinase K and incubated for 3−16 h at 37°C. They were extracted once with phenol−chloroform−isoamyl alcohol (12:12:1), once with chloroform, and treated for 1 h at 47°C with 100 μg/ml RNase A. The samples were again extracted once with phenol−chloroform−isoamyl alcohol, twice with chloroform, and ethanol precipitated. The resulting pellets were resuspended in 10 mM Tris−HCl, pH 7.4, 2.5 mM EDTA. For digestion of naked DNA, 500 ng of plasmid was treated with the indicated amounts of enzyme in 1 ml of wash buffer containing 1 mM $CaCl_2$ and 60 μg/ml yeast tRNA. After 5 min at 25°C, the reaction was stopped by addition of 100 μl EDTA, 10 mM EGTA, and the DNAs immediately purified as described above.

MPE-Fe(II) cleavage

Nuclei were prepared as described above and resuspended in cleavage buffer (15 mM Tris−HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.25 M sucrose) at a final OD_{260} of 30. MPE-Fe(II) cleavage was performed as described by Cartwright *et al.* (1984) with the following modifications. MPE-Fe(II) was prepared by mixing equal volumes of 1 mM MPE and 1 mM ferrous ammonium sulfate and rapidly diluting it 1:10 in cleavage buffer. The cleavage reaction contained the nuclear suspension (0.2 or 0.5 ml), 2 mM H_2O_2 and 2 mM dithiothreitol (DTT). The reaction was carried out in 1 ml, and was initiated by the addition of the MPE-Fe(II) to a final concentration of 10 or 25 μM. In some experiments, the H_2O_2 was omitted (as indicated in the figure legends). The samples were incubated at 25°C for periods up to 1 h. The reaction was stopped by addition of 100 μl of 500 mM bathophenanthroline. One ml of a solution containing 25 mM EDTA, 2% SDS and 200 μg/ml proteinase K was added and the samples incubated for 3−18 h at 37°C. The samples were then extracted once with phenol, once with chloroform and ethanol precipitated. The pellets were washed carefully with aqueous ethanol to remove all MPE, resuspended in 2 ml of TE buffer, treated for 1 h at 37°C with 100 μg/ml RNase A, and re-extracted once with phenol−chloroform−isoamyl alcohol (12:12:1), twice with chloroform and ethanol precipitated. Naked DNA was purified from nuclei and cleaved under the same conditions as the chromatin reactions. It was recovered by ethanol precipitation and used without further purification.

DNase I digestion

Nuclei were prepared as described above, diluted to an OD_{260} of 10−20 in wash buffer, distributed in 1 ml fractions, adjusted to 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and held on ice. The reaction was initiated by addition of the enzyme (Worthington−Cooper, 2000−2600 U/mg; 0.1 mg/ml solution) and the tubes incubated at 37°C. After 10 min the reaction was stopped by addition of 1 ml of a 25 mM EDTA, 2% SDS, 200 μg/ml proteinase K solution. DNA was isolated as described for samples digested with micrococcal nuclease.

Indirect end-labelling analysis

Purified DNAs were restricted to completion with the appropriate enzymes under conditions specified by the supplier. Samples were electrophoresed on 1−2%

agarose gels in Tris−acetate buffer, and the DNA was transferred to nitrocellulose or 0.2 micron Nytran membranes (Schleicher and Shuell) according to Southern (1975). The membranes were then hybridized with the nick-translated probes (sp. act. = $2-4 \times 10^8$ d.p.m./μg) as described in Figure 1. In some experiments using P4-A probe, the DNA was restricted with *AvaI* instead of *PstI*. The *AvaI* site is located 13 bp downstream from the MMTV LTR *PstI* site.

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